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(54) Title: AGGLUTINATION TEST FOR MYCOBACTERIAL ANTIGENS IN BIOLOGICAL SAMPLES

(57) Abstract

An agglutination test for detecting mycobacterium in a sample obtained from a biological fluid is provided. The test involves contacting the sample with particles coated with a monoclonal antibody directed towards a mycobacterium under conditions that permit agglutination visible to the naked eye when less than 10,000 bacteria per ml of biological fluid are present. Preferably the agglutination is enhanced with an enhancer provided at a concentration just below the minimum concentration of enhancer that allows autoagglutination of the particles detectable with the naked eye in the absence of an antigen. Also provided are kits and kit components for performing the agglutination test.

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AGGLUTINATION TEST FOR MYCOBACTERIAL ANTIGENS IN BIOLOGICAL SAMPLES

Field of the Invention

This invention relates to agglutination methods useful for detecting analytes in biological samples. The invention is particularly useful for detecting mycobacteria in a sample from an individual suspected to have or being tested for tuberculosis.

Background

The mycobacteria are a diverse assemblage of acid-fast, Gram-positive bacteria, some of which are important disease-causing agents in humans and animals, Bloom et al., Rev. Infect. Dis., 5:765-780 (1983); Chaparas, CRC Rev. Microbiol., 9:139-197 (1982). In man, the two most common diseases caused by mycobacteria are tuberculosis and leprosy, the causative agents being Mycobacterium tuberculosis and Mycobacterium leprae, respectively.

Other mycobacterial species are capable of causing tuberculosis or tuberculosis-like disease. Wallace, R.J., et al., Review of Infectious Diseases, 5:657-679 (1984). Mycobacterium avium, for example, causes tuberculosis in fowl and in other birds. Members of the M. avium-intracellulare

(MAI) complex are pathogenic among individuals with acquired immuno-deficiency syndrome (AIDS), as well as among other individuals having a compromised immune system. The members of the MAI-complex are resistant to standard anti-tuberculosis drugs. Pitchenik, A.E., et al., Annals of Internal Medicine, 101:641-645 (1984).

At present, nearly all tuberculosis results from respiratory infection with M. tuberculosis. Infection may often be asymptomatic, but could result in disease, producing pulmonary or other lesions which lead to severe debilitation or death. Today, tuberculosis remains a significant health problem, especially in developing countries. Worldwide, an estimated 11 million people are affected by the disease and about 3.5 million new cases occur each year. U.S. Congress, OTA, "Status of Biomedical Research and Related Technology for Tropical Diseases", OTA-H-258, Washington, D.C. 1985. Further, certain groups of individuals such as those who are HIV-positive have a markedly increased incidence of tuberculosis. Early diagnosis of TB is particularly important because the disease is preventable, treatable and curable.

Current diagnostic measures for these mycobacterial diseases are barely adequate. Efficient patient management and control of

transmission are compromised by current inadequacies in techniques for the rapid identification of the etiologic agent in the laboratory. Although bacilli may be detected by microscopy, Shoemaker, S.A., et al., Am. Rev. Respir. Dis., 131:760-763 (1985), intact bacilli are required and sensitivity is low. Samples are therefore cultured to allow for more accurate diagnosis as well as to permit definitive species identification. Vestal, A.L., HEW Publ. No. (CDC)77-8230 Atlanta, 1975; Bates, J.H., Am. Rev. Respir. Dis., 132: 1342 (1985). However, M. tuberculosis is difficult to culture and has a generation time of 15-20 hours. Wayne, L.G., Am. Rev. Respir. Dis., 125 (Suppl.) 31-41 (1982). A delay of up to 6 weeks before results of laboratory tests are available is not unusual.

Due to the disadvantages of using a diagnostic method reliant on microscopic observations, immunoassays have been studied for their applicability in diagnosing TB. Several immunological methods for detecting mycobacterial antigens such as enzyme linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) have been mentioned as possible alternatives to microscopy (Daniel, Reviews of Infectious Diseases Vol II Supplement 2, March-April, 1989, pp.S471-S478).

Cambiaso et al. (Journal of Immunological

Methods 129 (1990), pp. 9-14) describe an immunoassay for detecting mycobacterial antigens using a latex agglutination assay. In the method of Cambiaso, the latex particles are coated with a polyclonal antiserum reactive with mycobacterial antigens. Agglutination was detected with an optical counter, and apparently was not detectable by visual observation with the naked eye.

The use of polyclonal antisera is typical in latex agglutination assays, but has drawbacks. The principal drawback is that the use of polyclonal antiserum does not permit standardization. Different batches of polyclonal antisera will have different properties. Monoclonal antibodies have been reported in connection with latex agglutination assays in only a few instances. Monoclonal antibodies are reactive with a single antigen, and it is problematic to obtain a monoclonal antibody to a bacillus with sufficient specificity and titer so as to permit agglutinaton in such an assay.

U.S. Patent 4,965,192 to Maes, issued October 23, 1990, characterizes an antigen (A60) of mycobacterium, and reports the use of monoclonal antibodies against A60 in a latex agglutination assay. The '192 patent underscores the drawbacks of the prior art. According to the '192 patent, 100,000 bacteria/ml of sputum was needed "to begin

to see an aggregation". This level of detection is unacceptable for clinical diagnostic purposes. Conventional microscopy, which is considered to have low sensitivity, can detect 10,000 intact bacteria/ml of sputum. Although microscopy detects bacteria in only 40-60% of diseased individuals when uncultured sputum is used as the sample, it nevertheless is the best method currently available and is an order of magnitude better than the agglutination assay of the '192 patent. Moreover, the '192 patent did not use sputum from diseased individuals. Rather, cultured mycobacteria were mixed with the sputum of non-diseased donors. Thus, the ability of the '192 agglutination assay to detect mycobacteria in the sputum (which includes non-intact bacteria) of diseased individuals remains suspect.

Summary of the Invention

It thus is an object of this invention to provide a quick, easy method useful in the diagnosis of diseases caused by mycobacteria, e.g., tuberculosis or leprosy.

It is another object to this invention to provide a detection method capable of being performed without the use of detection accessories such as a microscope or scintillation counter.

It is still another object of the invention to provide a diagnostically useful agglutination assay which uses particles coated with a monoclonal antibody.

It is yet another object of the invention to provide an agglutination assay that does not necessarily require intact bacteria for detection.

According to one aspect of the invention, there is provided a rapid, reliable method for detecting mycobacteria, including the causative agent responsible for tuberculosis. This method is capable of detecting mycobacterial antigen in biological samples, including sputum, at levels that are clinically acceptable. This method may be conducted and the results evaluated by a person having limited expertise. The person only has to be capable of identifying the presence or absence of agglutination.

The method includes contacting the biological sample with particles coated with antibody directed towards a mycobacterial antigen. Preferably, the antibody is a monoclonal antibody. The biological sample is contacted under conditions which permit agglutination to occur. After a short period of time, the agglutination of the particles is detected as an indication of the presence of mycobacteria in the biological sample. Preferably, conditions are

applied such that the agglutination is visually detectable. The method permits detection at clinically acceptable levels, that is, 10,000 bacteria/ml of biological fluid, and more preferably permits detection in samples obtained from fluids having levels of 1000 bacteria/ml.

According to another aspect of the invention, an agglutination reaction is enhanced with an enhancer. The enhancer is a reagent capable of promoting the agglutination reactions preferably in a manner which assures that agglutination will be visually detectable by the naked eye if the appropriate antigens are present in the biological sample.

This invention also pertains to particles coated with monoclonal antibodies which may be used in the above-described methods and kits containing the reagents necessary to conduct the methods of the invention.

Another aspect of the invention pertains to the use of a negative control latex which allows one to differentiate if latex agglutination is due to antigen-antibody interaction (specific agglutination) or whether something else in the sample causes non-specific agglutination with the antibody coated on the latex beads.

Brief Description of the Drawing

Figure 1 is a graph used in selecting monoclonal antibodies for the method(s) of this invention;

Figure 2 is a table illustrating the relationship between the concentration of enhancer and autoagglutination;

Figure 3 illustrates a preferred kit according to the invention; and

Figure 4 shows a slide of the kit of Fig. 3.

Detailed Description

The agglutination method of the invention involves contacting a biological sample with a particle coated with an antibody. The antibody is specific towards an analyte being tested for in the sample and may include as equivalents any agent capable of binding to the analyte. Agglutination is detected as an indication of the presence of the analyte in the sample. A monoclonal antibody may be used, and both the contacting and detection steps of the invention preferably are conducted within a relatively short period of time, e.g. preferably within about fifteen minutes.

According to one aspect of the invention, the antibody is directed towards an analyte indicative of a mycobacterium. Most preferably the antibody is

a monoclonal antibody directed toward an antigen from a mycobacterium. According to this aspect of the invention, the sample is contacted with particles coated with a monoclonal antibody directed towards a mycobacterial antigen under conditions which permit agglutination to occur. The agglutination of the particles is detected as an indication of the presence of mycobacteria in the sample. Examples of mycobacteria which may be detected include Mycobacterium tuberculosis and Mycobacterium leprae.

The mycobacterial antigen detected may be lipoarabinomannan (LAM) antigen. LAM is a highly immunogenic lipopolysaccharide. LAM is a prominent component of the cell walls of both M. leprae and M. tuberculosis and has been implicated as a major B cell stimulant in tuberculosis and leprosy. Portions of LAM are exposed on the surface of mycobacteria.

The particles used in this invention may be any particles capable of agglutinating in a detectable manner. The prior art has disclosed particles typically in a size range of from about 0.1μ to about 15μ; however, larger and smaller particles may be used. A preferred size particle for this invention is 0.15μ - 1.0μ, more preferably about 0.2μ.

The type of particles used within this

invention include red blood cells, glass beads, liposomes, pollen spores, metal oxide particles, latex, and carbohydrates, e.g. dextran, agarose or cellulose. The preferred particles are latex particles.

The term latex is art recognized and typically refers to particles made of natural or synthetic rubber or plastic. Latex particles are commercially available and are prepared using addition polymerization processes in aqueous media. Monomers used in preparing latex include acrolein, acrylate, methyl acrylate, methacrylate, methyl methacrylate, glycidyl methacrylate, styrene, vinyl toluene, t-butyl styrene and copolymers containing mixtures of these monomers. The polymers and copolymers optionally may contain cross-linking agents such as divinyl benzene and butadiene.

The particles also may be colored, thereby enhancing the ease of visual detection of agglutination. The color may be selected to provide a contrast between the particles and the background color of a slide, for example. The preferred particles of this invention are red latex beads which can be obtained from Rhone Poulenc, France. The red beads are polystyrene having an average diameter of about 0.2μ. Such beads provide a desirable contrast against an opaque slide that is white.

The term "coated particle" is intended to encompass a particle having antibody on its surface. The surface of a particle may be coated using methods capable of directly or indirectly attaching antibodies. The antibodies may be absorbed directly on the surface of the particle or attached to the particle through a spacer molecule, e.g. a molecule capable of bonding to both the surface of the particle and to the antibody. Preferably, the antibodies are directly attached or absorbed to the particle using passive coating techniques well known to those of ordinary skill in the art. Such coating techniques tend to preserve the specificity and activity of the immunological reagent.

The antibodies of this invention are preferably those directed towards mycobacterial antigen. Directed towards is intended to encompass antibodies capable of binding with an antigenic portion of a mycobacterium. The term antibody is intended to include whole antibodies, antibody fragments, chimeric antibodies containing portions from two different species, and synthetic peptides identical to or functionally nalogous to the antibody. Antibody fragments such as $F(ab')_2$, Fab and F_v may be produced by standard techniques of enzyme digestion. In addition, synthetic peptides

representing Fab and F_v analogues can be produced by genetic engineering techniques. See e.g., Better, M. et al. (1988) Science 240:1041; Huston, J.S. et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883.

The preferred form of antibody is whole, monoclonal antibody. It should be understood that more than one type of monoclonal antibody may be attached to a particle. A particularly preferred monoclonal antibody for one embodiment of the invention is designated ML9D3, produced by the ML9D3 cell line, ATCC Accession No. HB 10684, Rockville, Md. This monoclonal antibody is directed toward the LAM antigen.

Examples of monoclonal antibody-producing cell lines include hybridoma cell lines, myeloma cell lines, or viral or oncogenically transformed lymphoid cells. Hybridoma cells which can produce the specific antibodies for use with this invention may be made by the standard somatic cell hybridization technique of Kohler and Milstein, Nature 256:495 (1975) or similar procedures employing different fusing agents. Briefly, the procedure is as follows: the hybridoma which secretes the monoclonal antibodies are produced by immunizing an animal with an antigen. Lymphoid cells (e.g. splenic lymphocytes) are then obtained

from the immunized animal and fused with immortalizing cells (e.g. myeloma or heteromyeloma) to produce hybrid cells. The hybrid cells are screened to identify those which produce the desired antibody and then are cloned and tested to prove that the cells produce only monoclonal antibodies. The hybridoma cells producing the desired antibody can be subsequently expanded. The hybridomas are expanded by injecting them intraperitoneally into mice under conditions which allow ascites fluid to develop. The ascites fluid is collected from the mice, pooled together and centrifuged. The supernatant from this process is termed neat ascites.

The conditions which permit agglutination to occur are those which lead to the agglutination of the particles in a manner which is detectable, preferably visually with the naked eye. For this, approximately 100 clumps must be seen to determine agglutination. Clumps must be about 50μ in size to be visible with the naked eye. Approximately 10 bonds are required per particle to cause clumping. The conditions should be such that agglutinaton is detectable at levels of 10,000 bacteria/ml of biological fluid, and preferably at levels of 1000 bacteria/ml of sputum. This will ensure detection of greater than 50% of those individuals having a mycobacterial disease. Examples of such conditions

include the selection and density of the antibody coated on the particles and the use and selection of an enhancer.

A selection process such as an ELISA assay may be used to determine whether a particular lot or batch of monoclonal antibodies would be useful in the method of this invention if coated on the particles. In an ELISA assay, 1 µg/ml of LAM antigen can be coated on a solid phase, e.g. multiwell plate. LAM may be prepared as described below in Example 6 and in copending U.S. application serial no. 07/654,321, filed February 12, 1991, and entitled "Purified LAM and Synthetic Analogs Thereof", the entire disclosure of which is incorporated herein by reference. The coated solid phase then is incubated with various dilutions of monoclonal antibodies at 37°C for approximately one hour. The unbound monoclonal antibodies are separated from the solid phase. The solid phase with attached antibodies is subsequently incubated with a second antibody directed towards the monoclonal antibody and conjugated to an enzyme (incubation with secondary antibody is for 1 hr at RT). Subsequently, the solid phase-antibody-secondary antibody conjugate is incubated for an additional hour at 37°C in the presence of the substrate for the enzyme. The

enzymatic reaction is measured using spectrophotometric means, e.g. absorbance, as an indication of the amount of monoclonal antibody bound to the antigen. A curve may be established by plotting the absorbance vs. dilution and the "titer" of the monoclonal antibodies may be determined (Figure 1).

The quantitation of specific activity or titer is expressed as the reciprocal of the dilution of monoclonal antibody which exhibits 50% of maximum absorbance. This can be determined from a curve established by plotting the % of maximum absorbance of particular dilutions of monoclonal antibodies versus the dilution of the monoclonal antibodies (MAb) as shown in Figure 1. The % of maximum absorbance of each dilution can be calculated by using the following formula:

$$\frac{B-NSB}{Bo-NSB} \times 100$$

B = read out absorbance of each dilution of antibody
Bo = maximum absorbance, which is considered 1.000
NSB = read out absorbance of nonspecific binding

For example, if at a dilution 1:30,000, the readout absorbance (B) is 0.783(B), and the nonspecific absorbance (NSB) is 0.110, and the maximum absorbance (B0) is always considered 1.000 (100%

absorbance), the % of maximum absorbance of MAb at this dilution then is calculated as follows:

$$1{:}30{,}000 = \frac{0.783-0.110}{1.000-0.110} \times 100 = 75.6\%$$

Following the procedure of the above ELISA and using ML9D3, the dilution which showed 50% of maximum absorbance was 1:85,000, (Figure 1) which is inversely proportional to the titer of that particular batch of monoclonal antibodies. Thus, the titer is 85,000 Ab units/unit volume (85K).

If the solution has a titer of antibodies against LAM of at least 70K [approximately 3 mg antibody per ml], and more preferably 100K [approximately 5 mg antibody per ml], at 50% absorbance, then the lot or batch used in preparing the dilutions may be used to coat particles for use in this invention. It should be understood that the foregoing titers are merely cut-off limits useful for deciding whether a lot or batch of antibodies is suitable for coating particles according to this invention.

Other methods may be used to determine the suitability of various lots or batches of antibodies and also to improve lots or batches of antibodies such that they are useful within this invention.

For example, even if the titer of antibodies is less than 70K at a 50% absorbance measured using the method described above, the solution still may be useful in the agglutination assays of the invention after various manipulations, e.g. purification and/or concentration processing steps. Problems may be encountered if attempting solely concentration of the antibodies because the other components of the ascites, enzymes etc., also become concentrated. Concentration alone thus may lead to nonspecific binding and a decrease in stability. However, concentration coupled with purification may be desirable not only to attain adequate titer, but also to increase the stability of the monoclonal antibodies.

The amount of monoclonal antibody coated on the particles is dependent on a number of variables. In one preferred embodiment, 100 µl of neat ascites having a titer of at least 100K [approximately 5 mg antibody per ml] of antibodies against LAM at 50% absorbance, i.e. a monoclonal antibody selected using the cut-off limits described above, is combined with 4 ml of a 0.5% suspension of latex beads (0.2µ polystyrene). It should be understood that these parameters are interrelated. For example, if the volume of neat ascites is increased, then a larger volume of solution containing latex

beads or a solution of latex beads having an increased concentration of beads should be used.

The monoclonal antibody preferably is present on the surface of the particle at a density sufficient such that the particles are capable of autoagglutinating in a manner detectable with a naked eye in the presence of an enhancer (discussed in greater detail below) at a preselected concentration. Preferably, a preselected concentration of enhancer is used as a standard, and only coated beads capable of autoagglutinating as seen by the naked eye in the presence of this preselected concentration of enhancer are used. It should be understood that this concentration of enhancer is used for selection purposes only. It is not the same concentration of enhancer actually used in the agglutination method of this invention. The concentration of enhancer used in the agglutination method of the invention is selected as described below.

Enhancers are additives which promote nonspecific agglutination generally. Thus, an enhancer at sufficient concentration may be added to a solution containing antibody-coated beads and thereby cause agglutination of the coated beads without the addition of antigen. This ability to promote agglutination can be exploited to turn a

weak agglutination reaction visible only using instrumentation (e.g. microscopic or optical means) into one visible to the naked eye. To do this, the concentration of enhancer is selected to be just below that which will cause autoagglutination of the coated beads in the absence of antigen. Under these conditions, very little antigen is necessary to trigger a level of agglutination visible to the naked eye. Examples of enhancers include polyethylene glycol (PL , mol. wt. 8000), polyvinylpyrrolidone (PVP, mol. wt. 40,000, K value or intrinsic viscosity of 29-32), dextran (mol. wt. 70,000) and combinations thereof.

The concentration of enhancer used in the agglutination assays may be selected as follows. Various concentrations of the enhancer are prepared. At the higher concentrations, the enhancer typically will cause autoagglutination of the coated particles visible to the naked eye in the absence of antigen. Lower concentrations are tested until no visually detectable autoagglutination occurs. The concentration of enhancer immediately below the minimum concentration causing visually detectable autoagglutination preferably is selected for use in the method of this invention. Enhancers selected in this manner are capable of providing autoagglutination visible to the naked eye when a

biological sample containing the appropriate antigen is part of the agglutination reaction mixture.

The agglutination of the particles may be detected by using any means capable of measuring the agglutination of the particles in a quantitative or qualitative manner. The preferred means of detection is visual detection with the naked eye. Detection may also be carried out using the aid of a microscope or other optical instruments, i.e. optical counter. However, when appropriate concentrations and conditions as set forth above are selected, detection aids are unnecessary and the test thus is greatly simplified and economized.

The biological samples used within the method of this invention may be any biological sample in which agglutination is detectable. It may include, for example, viable bacteria, killed or fragments of bacteria and/or soluble antigen derived from bacteria, depending upon the preferred processing method employed. For safety considerations, it may be desirable to inactivate the bacteria by processing procedures such as bioling in NaOH, which particular procedure would result in substantial solubilization of the bacteria.

The type of biological sample selected may depend on the type of disease suspected or the condition of the individual being tested. For

example, genital specimens (e.g. uterocervico-vaginal secretions) or urine may be obtained from individuals suspected of having urogenital tuberculosis. Cerebrospinal fluid may be obtained if tuberculous meningitis is suspected. Examples of biological samples include serum, whole blood, urine, feces, tissue specimens, (e.g. pus, exudates and biopsy specimens), cold abscess drainage, peritoneal ascitic fluid, uterocervicovaginal secretions, cerebro-spinal fluid, pulmonary secretions, (e.g. bronchoalveolar and gastric lavage), pleural fluid and sputum.

Mycobacteria may infect almost any tissue of the body. The successful detection of the bacteria may depend on the techniques used in collecting and processing the biological samples. The processing techniques typically are either decontamination and/or concentration procedures, which kill or reduce to negligible levels all bacteria other than mycobacteria and/or which concentrate the number of mycobacteria per volume of sample. Examples of types of decontaminaton and/or concentration processes which can be used include the N-acetyl-L-cysteine-(NALC) sodium hydroxide, benzalkonium chloride (zephiran) trisodium phosphate, and sputolysin-oxalic acid methods. Versions of these methods are art recognized. O.

George W. Berlin, "Mycobacteria", Bailey and Scott's Diagnostic Microbiology, 5th edition p. 597-640. Autors. Ellen Jo Baron and Sydney M. Finegold. The preferred method is described in more detail in Example 5 below.

Some biological samples may require special pretreatment or handling prior to being decontaminated and/or concentrated. Gastric lavage should be processed immediately or neutralized with a basic solution (e.g. 10% sodium bicarbonate) and refrigerated until processed as with sputum. If more than ten ml of watery-appearing aspirate is obtained, centrifugation may be appropriate (e.g. 3600 x g for 30 minutes) and only the sediment saved for processing in the decontamination and/or concentration steps.

Urine samples can be divided into about four volumetric aliquots, e.g. 50 ml, and centrifuged to form a sediment or pellet (e.g. 3600 x g for 30 minutes). The supernatant fluid may be decanted. The combined sediment then may be collected, brought up to 10 ml and used in the decontamination and/or concentration steps (preferably as in Example 5).

For feces, one or two grams of formed stool or 5 ml of liquid stool can be transferred to a 50 ml centrifuge tube and distilled water added to bring the volume up to 10 ml. The suspension is vortexed

thoroughly. The specimen then is filtered through gauze to remove particulate material. Ten ml of NALC-NaOH can be added to the suspension and allowed to stand at room temperature for 45 to 60 minutes. Then, 25 ml of phosphate buffer is added, mixed thoroughly, and centrifuged for 20 minutes at 3,600 X g. The supernatant fluid is decanted and the sediment resuspended and assayed according to the invention.

Pus and wound aspirates can be transferred to a 50 ml centrifuge tube with 10 ml distilled water. The specimen is vortexed vigorously and allowed to stand for 20 minutes; then the suspension is processed as with sputum.

Pieces of tissue thought to be contaminated are finely minced using a tissue grinder tub and pestle. Ten ml of distilled water is added, vortexed vigorously, and then allowed to stand for 20 to 30 minutes. The material then is transferred to a 50 ml centrifuge tube. An equal volume of NALC-NaOH is added and then mixed vigorously and allowed to stand for 20 minutes. Twenty-five ml of phosphate buffer is added. The tube is mixed vigorously and centrifuged at 3,600 X g for 20 minutes. The supernatant fluid is decanted and the pellet is suspended in 100 to 200 µl Tris buffered saline and used in the assays of the invention.

Cerebrospinal fluid may be centrifuged to concentrate the bacteria and the supernatant fluid discarded. The pellet containing the bacteria may be resuspended in distilled water or in an aqueous solution with thorough mixing and the mixed solution may be used in the agglutination methods of the invention.

Pleural fluid may be collected in sterile anticoagulant (e.g. in the presence of ethylene diamine tetraacetic acid or heparin), centrifuged to form a pellet of concentrated bacteria and the supernatant fluid discarded. The pellet may be resuspended in distilled water or an aqueous solution and the mixed solution may be assayed according to the invention. If the pleural fluid becomes clotted, it may be liquified using sputolysin and/or vigorous mixing. Preferably, a pellet from 20 ml of pleural fluid is resuspended in 50 to 100 microliters of buffer and then assayed according to the invention.

Blood may be collected in sterile anticoagulant. The blood may be allowed to stand at room temperature until separation of the plasma and other blood components has occurred. The leukocyte-rich plasma may be removed and centrifuged at 400 x g until a pellet is formed (e.g. 15 minutes). The pellet may be resuspended under

conditions that allow cell lysis (e.g. in a cell lysing agent such as ammonium chloride). The lysed cells may then be centrifuged (3600 x g) until a pellet is formed (e.g. 30 minutes). The pellet may be resuspended in a buffer solution (preferably 100 - 200 µl) and used in the agglutination methods of the invention.

Sputum may be processed by mixing with a basic solution, often a NaOH-NALC solution. The combination of the basic solution and the sputum may be mixed thoroughly, kept for 15 min., and subsequently centrifuged. The supernatant fluid may be decanted and a buffer having a pH of about 6.5 may be added to the pellet. The pH of the sputum may be tested with pH paper to determine whether the sputum is substantially neutral. The details of processing sputum are set forth in detail in Examples 5, below.

This invention also pertains to kits useful for testing biological samples of individuals for the presence of mycobacteria. The preferred kits 10 (Fig. 3) include a plastic container 12 containing particles coated with a monoclonal antibody directed towards mycobacterial antigen. The monoclonal antibodies preferably are the same as those described above. The kit 10 further may have an enhancer as described above and preferably in a

separate container 14 at a preselected concentration. The kit further may contain slides having a designated region for performing the assays of the invention. For example, Fig. 4 shows an opaque, white slide 16 having a region 18 designated by a circle drawn on the slide. The region 22 also may be designated by other means such as a recessed region. The kits also may include at least one reagent container 20 including reagents for processing a biological sample. The kits further may include other containers for positive or negative control reagents, and preferably includes a container for negative control latex. Finally, the kit may include instructions related to the use of the kit in aiding in the diagnosis of an individual suspected of having or being tested for tuberculosis.

This invention will now be further illustrated using the following non limiting examples:

Example 1 - Passive Coating of Latex Beads (Adsorption) with Monoclonal Antibody Anti-LAM

A 15 ml polystyrene centrifuge tube was blocked by delivering six ml of a blocking reagent [3% milk caseine, 5% fetal calf serum in coating buffer (20 mM Tris-HCl, 0.15 M NaCl, pH 7.5)] to the tube and

incubating for one hour at room temperature while mixing in a rotator. The blocking reagent was removed and the tube was washed twice with six ml of saline. Two ml of a solution of 1% latex red beads (0.2 micron average diameter, Rhone Poulenc, France) in coating buffer was then delivered to the tube. An additional four ml of coating buffer was delivered to the tube and the mixture was centrifuged in the cold (4°C) at 27,000xg for fifteen minutes.

The supernatant fluid was discarded using vacuum aspiration and the pellet formed during centrifugation was resuspended in two ml of coating buffer. The suspension was vortexed vigorously (and/or pipetted back and forth) to disperse the latex particles. An additional four ml of coating buffer was added and the suspension was centrifuged in the cold again at 27,000xg for fifteen minutes. The supernatant fluid was discarded and the pellet was washed once more as described above.

The washed pellet was resuspended in two ml of coating buffer and the latex beads were dispersed in the coating buffer using a pensonicator. The homogeneity of the latex beads was confirmed by microscopic examination. The suspension was delivered to the previously blocked polystyrene tube and an additional 2 ml of coating buffer was added.

One hundred µl of neat ascites containing anti-LAM monoclonal antibody (neat murine ascites with a 100K titer of ML9D3 antibody as determined by ELISA assay, and stored at -20°C) was added to the latex suspension and vortexed for two seconds. The tube was mixed on a flying rotator for four hours at room temperature. The latex suspension then was transferred into a 6 ml centrifuge tube and was spun down at 27,000xg for fifteen minutes. The resulting pellet was resuspended in two ml of assay buffer (same as coating buffer) and vortexed to get a good dispersion. Then 4 ml of assay buffer was added, followed by centrifugation at 27,000xg for fifteen minutes. The pellet was resuspended in 2 ml of a second blocking reagent (1% BSA, 5% glycerol and 1% sodium azide in coating buffer), and maintained at room temperature for 30 minutes to block all the reactive sites on the bead surface.

After the foregoing procedure, the latex beads are ready to be examined for the presence of antibody on their surface and for the specific activity of these antibodies.

As stated earlier in the specification, the neat ascites may be purified prior to sensitizing the beads. This may be desirable because it increases the stability of the antibody coated beads in relatively high temperature conditions (e.g.

37°C). Procedures such as ammonium sulfate precipitation (at 50% saturation) or Protein A chromatography have been used successfully to purify the neat ascites. Then, the procedure for coating the beads is carried out as outlined above with the following exceptions: 4 ml of 0.5% washed latex beads in coating buffer is sensitized with 0.6 ml of a mixture of 100 µl purified MAb (having 100K titer [approximately 5 mg antibody per ml]) and 500 µl 1% BSA. The mixture of MAb and BSA preferably are kept for 20 minutes at RT for stabilization before adding to latex beads.

Example 2 - Examination of the Presence of Antibody on Sensitized Latex Beads

Twenty µl of assay buffer (20m M Tris-HCl, 0.15M NaCl, ph 7.5), 40 µl of anti-mouse IgG (1:100) and 20 µl sensitized beads were delivered to the surface of a slide. The components were mixed with a mixing stick and the slide was left on a mechanical rotator for several minutes. A positive reaction is indicated by the appearance of large clumps of red beads, indicating the presence of the antibody on the surface of the beads. A negative control consisting of 60 µl assay buffer and 20 µl sensitized red beads was delivered and tested using the same procedure. No agglutination was detectable

Example 3 - Selection of Concentration of Enhancer

An enhancer (PEG) is dissolved in saline (0.8% NaCl in deionized water) at concentrations ranging from 7% to 0.5%. These various concentrations of enhancer are run in a standardized agglutination assay to determine the minimum concentration of enhancer which causes agglutination. Then, the concentration below that which results in agglutination visible to the naked eye is selected as that for use in the assay of Example 5.

To select the proper concentration of enhancer, 20 microliters of a defined concentration of enhancer, 40 microliters of assay buffer and 20 microliters of sensitized latex beads are delivered to a slide. These reagents are mixed on the surface of the slide, and the slide is rotated on a mechanical rotator for 5 minutes. Then, it is determined whether autoagglutation has occurred. Autoagglutation is considered to be present when the agglutination is visible to the naked eye. The test is repeated for various concentrations of enhancer. The results are shown in the table of Figure 2. A plus sign represents agglutination detectable with the naked eye.

The enhancer concentration selected for use in the agglutination assay of the invention is chosen as that concentration below, but close to, the

minimum concentration of enhancer that shows autoagglutination. In this example, that concentration either is 4.0% enhancer or 3.5% enhancer. Four % enhancer would provide a slightly more sensitive assay than one using an enhancer concentration of 3.5%.

Example 4 - Examination of the Specific Activity of the Sensitized Latex Beads

An acetone precipitate of M. tuberculosis for use as a positive control in the latex agglutination test was prepared. Mycobacterium tuberculosis strain TMC 107 (Erdman) is grown for eight weeks in a glycerol-alanine-salts medium as a shaker culture. The Erdman strain was obtained from the Trudeau Mycobacterium Culture Collection, Trudeau Institute, Saranac Lake, NY, culture number TMC 107 and is available at the ATCC, No. 35801, Rockville, Md., U.S.A. Other strains may be employed, including the rapid growing, attenuated strain of M. tuberculosis H37Ra, obtained from K. Takayama, Madison, WI, described by Takayama, K., et al. (1975), J. Lipid Res., 16, 308-317 and available from the ATCC, No. _____.

The cultures are autoclaved at 80°C for 1 h, cooled and filtered. The harvested cells are washed several times with distilled water and stored frozen (-20 C) until ready for breakageN Harvested cells (~130 g wet weight) were resuspended in PBS

containing 0.5% Triton X100 and 0.02% NaN_3 (200 ml). A thick suspension is desirable in order to achieve complete breakage of cells. LAM, lipomannan (LM) and phosphoinositol mannoside (PIM) have a great affinity for detergent. Use of Triton X100 when breaking the cells helped to keep most of these amphipathic molecules in solution thereby giving maximum yield during acetone precipitation.

The suspension was sonicated while cooling in an ice bath for 10 min with a W-385 Sonicator Ultrasonic Liquid Processor (Heat Systems-Ultrasonic, Inc., Framingdale, NY) operating at optimal cavitation intensity. The sonicate was passed four times through a French pressure cell (Model SA073; American Instruments Co., Urbana, IL) at 20,000 lb sq. in. The sonicate pressate was centrifuged twice at 27,000 x g for 45 min. The pellet was washed twice with the above buffer (50 ml each time) and recentrifuged. The supernatant fluids were combined and recentrifuged (at 27,000x g) in order to remove most of the cell wall. (The supernatant fluid appeared translucent after centrifugation.)

To the precooled supernatant fluid, distilled acetone was added (to a final concentration of 90% acetone) to precipitate mainly polysaccharides. Some proteins were also precipitated during this

procedure. (Considering that very large volumes of solvents were used, it was more efficient when the supernatants were divided into two 1000 ml Erlenmeyer flasks.) The acetone precipitate was stored at 4°C for 48 h.

The precipitate was collected by centrifugation at 10,000 x g and air dried.

Twenty µl of enhancer (4% polyethylene glycol), 40 µl of an acetone precipitate of M. tuberculosis suspension (83 µg/ml) and 20 µl of sensitized red beads are delivered onto the surface of a slide and mixed with a mixing stick. The slide containing the mixture is placed on a mechanical rotator for five minutesN A negative control of 20 µl enhancer, 40 µl assay buffer [see Example II] and 20 µl sensitized red beads was subjected to the same procedure. The specific activity of the sensitized latex beads was determined by observing for agglutination of the red beads in the presence of the positive control (acetone precipitate of M. tuberculosis) and the absence of agglutination with the assay buffer.

Example 5 - Latex Agglutination Test for detection of Mycobacteria in Human Sputum

An equal volume of a solution containing 50 ml

of 1N (4%) NaOH and 50 ml of 2.9% trisodium citrate, H_2O and 0.5 g of N-acetyl-L-cysteine (NALC powder) is added to an equal volume of a sputum sample in a 50 ml centrifuge tube and vortexed thoroughly. Preferable at least 1 ml of sputum is present. The mixture is allowed to stand for fifteen minutes. The tube is filled up to the 45 ml mark with distilled water and centrifuged at 2800 x g to 3000 x g for fifteen minutes. Subsequently, the supernatant fluid is carefully decanted and 100-200 µl of PO_4^{3-} buffer (pH 6.8) is added to the pellet. Following sputum processing, the processed sputum is neutralized, if necessary, with neutralizing reagent (1 N HCl). The pH of the sputum to be used in the agglutination assay must be approximately pH 7 to allow appropriate physiological conditions for antibody-antigen binding to occur.

In the agglutination assay, 20 µl enhancer, 40 µl processed sputum sample, and 20 µl sensitized beads is delivered to a slide. The various reagents are mixed with a mixing stick and the slide is placed on a mechanical rotator for five minutes. The presence of agglutination is then detected.

A negative control latex is comprised of 20 µl enhancer, 40 µl of the same processed sputum,

and 20 µl of sensitized negative latex beads (mixed with sputum and enhancer). The negative latex beads are prepared as set forth in Example I, using neat ascites to coat the beads with the exception that the sensitized beads are then stored for at least 7 days at 37°C prior to use. This results in beads sensitized with monoclonal antibody lacking specific activity i.e. the beads will agglutinate with anti-mouse antibody, but not with a sample of positive control antigen or a sample containing mycobacteria. The negative control latex may also be obtained by sensitization of the latex beads with purified monoclonal antibody, but without the use of BSA as a coadsorbent. In this case also the beads bind to anti-mouse antibody, but lack specific activity.

EXAMPLE 6

Preferred Method of Generation and Purification of LAM from Mycobacterium tuberculosis

Mycobacterium tuberculosis strain TMC 107 (Erdman) is grown for eight weeks (or strain H37Ra for a lesser time) in a glycerol-alanine-salts medium as a shaken culture.

The cultures were autoclaved at 80°C for 1 h, cooled and filtered using sterile 0.22 micron

filtration system (Nalge Co., Rochester, NY). The harvested cells were washed several times with distilled water and stored frozen (-20°C) until ready for breakage. Harvested cells (~130 g wet weight) were resuspended in PBS containing 0.5% Triton X100 and 0.02% NaN_3 (200 ml). A thick suspension is desirable in order to achieve complete breakage of cells. LAM, LM and PIM have a great affinity for detergent. Use of Triton X100 when breaking the cells helped to keep most of these amphipathic molecules in solution, thereby giving a maximum yield during acetone precipitation.

The suspension was sonicated while cooling in an ice bath for 10 min with a W-385 Sonicator Ultrasonic Liquid Processor (Heat Systems-Ultrasonic, Inc., Framingdale, NY) operating at optimal cavitation intensity. The sonicate was passed four times through a French pressure cell (Model SA073; American Instruments Co., Urbana, IL) at 20,000 lb per sq. in. The sonicate pressate was centrifuged at 27,000 x g for 45 min, two times. The pellet was washed twice with the above buffer (50 ml each time) and recentrifuged. The supernatant fluids were combined and recentrifuged (at 27,000 x g) in order to remove most of the cell wall. (The supernatant fluid appeared translucent after centrifugation.)

To the precooled supernatant fluid, distilled acetone was added (to a final concentration of 90% acetone) to precipitate mainly polysaccharides. Some proteins were also precipitated during this procedure. (Considering that very large volumes of solvents were used, it was more efficient when the supernatants were divided into two 1000 ml Erlenmeyer flasks.) The acetone precipitate was stored at 4°C for 48 h.

The precipitate was collected by centrifugation at 10,000 x g and air dried. Dry precipitate (1 g) was suspended in 6 ml of 6 M guanidine HCl in 10mM Tris HCl, pH 7.4 by pansonication. (The insoluble material remaining is removed by low speed centrifugation (2000xg) prior to application to the column.) The soluble material is applied to a sephacryl S-400 column (1.5 x 150 cm) in the same buffer. Fractions (2 ml) are collected and monitored by PAGE. Fractions are pooled according to enrichment with LAM, LM and PIM and dialyzed extensively against water (5 to 6 changes of water). There is no resolution between LAM, LM or PIM after this preliminary column fractionation. The dialyzed fractions were freeze dried to yield approximately 500 mg of impure material.

Final purification of LAM was achieved by

applying the LAM, LM, PIM enriched fraction obtained from the S-400 column above to a Sephacryl S-200 column with a buffer containing 10mM Tris, 0.2 M NaCl, 1mM EDTA 0.02% sodium azide and 0.25% deoxycholate. About 150-180 mg of crude material was applied to a column size of (2.5 x 120 cm) and 4 ml fractions were collected and monitored by PAGE. Use of deoxycholate as a detergent on a simple sizing column keeps LAM, LM and PIM from aggregating; therefore, they purify rapidly as separate entities. This method of purification replaced several laborious and tedious ion exchange chromatography steps, as well as purification. Fractions containing pure LAM, LM and PIM (resolved at this stage) were pooled, dialyzed at 37°C for two days (48 hrs.) against the buffer without deoxycholate in order to remove detergent followed by dialysis against water at 4°C for two days (48 hrs.). Pure LAM, LM or PIM was stored as freeze-dried powder.

LAM has many LPS-like biological activities. To ensure that LPS contamination was not present in preparations, lyophilized LAM was redissolved in pyrogen-free water, filtered through 0.45 μm PTFE filtration unit and passed through 2.0 ml of Detoxi-Gel column (Pierce Chemical, Rockford, IL), refiltered through a second 0.20 μm sterile filter

and the filtrate collected into a sterile, pyrogen free vial using sterile pyrogen-free water to elute it off the gel.

As a means of quality control, all final preparations are subjected to 1) SDS-PAGE and silver stained with a periodate step to visualize the carbohydrates, 2) Western blot using the monoclonal antibody against LAM to verify its LAM content, and 3) Alditol acetate and GC analysis versus neutral sugar standards to estimate arabinose and mannose content.

Alternate Purification of LAM

The isolation of LAM-containing fractions from M tuberculosis and primary resolution on columns of DEAE-Sephacel in detergent-containing buffer, have been described (1,2). In addition to these steps, preparations of LAM, recovered from columns of DEAE-Sephacel and which were highly pure according to PAGE (1), were dialyzed, concentrated on an Amicon flow cell (10 kDa molecular weight cut-off membrane, Amicon model 8200; Danvers, MA), precipitated with 85% ethanol, and redissolved in 0.01M Tris HCl (pH 7.4) containing 0.1% Triton X-100 and applied to a HYDROPORE Ax HPLC column (21.4 mm x 25 cm, Rainin, Woburn, MA) equilibrated in the same buffer. The column was eluted with the same buffer

followed by a shallow gradient of 0 to 0.1M NaCl. Fractions (10 ml) were collected, analyzed for carbohydrate (2) and positive fractions re-examined by PAGE. Pure LM eluted with 0.01M NaCl, followed by PIM which eluted with 0.02M NaCl, and LAM which eluted with 0.05M NaCl. Fractions were pooled and subjected to a folch wash by treating with 6 parts Chloroform:methanol (2:1) and allowed to form a biphase. The aqueous layer was removed and dialyzed, concentrated and dried. LAM was reprecipitated with 85% ethanol at 0°C overnight and then centrifuged at 2000xg. To remove the last traces of detergent, a solution of pure LAM was passed through a column (2 ml) of Extracti Gel-D (Pierce Chemical Co., Rockford, IL) and eluted with H_2O.

EQUIVALENTS

Those skilled in the art will be able to ascertain, using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein.

These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for detecting mycobacteria in a sample obtained from a biological fluid comprising:

contacting the sample with particles coated with a monoclonal antibody directed towards a mycobacterial antigen under conditions permitting agglutination visible to the naked eye when less than 10,000 bacteria per ml of biological fluid are present: and

detecting agglutination of the particles as an indication of the presence of mycobacteria in the biological sample.

2. A method as claimed in claim 1 further comprising enhancing the agglutination with an enhancer.

3. A method as claimed in claim 1 or 2 wherein the monoclonal antibody is directed towards Mycobacterium tuberculosis.

4. A method as claimed in claims 1 or 2 wherein the monoclonal antibody is directed towards the LAM antigen.

5. A method as claimed in claims 1 or 2 wherein the particles are selected from the group

consisting of latex beads, red blood cells, glass beads, liposomes, pollen spores metal oxide particles and carbohydrate particles.

6. A method as claimed in claim 5 wherein the particles are latex.

7. A method as claimed in claims 1 or 2 wherein the biological sample is selected from the group consisting of serum, whole blood, urine, feces, tissue specimens, cold abscess drainage, semen, uterocervicovaginal secretions, cerebro-spinal fluid, bronchoalveolar, gastric lavage, sputum, pleural fluids, peritoneal tappings.

8. A method as claimed in claim 7 wherein the biological sample is sputum.

9. A method as claimed in claims 1 or 2 wherein the agglutination is detected visually with the naked eye.

10. A method as claimed in claim 2 wherein the enhancer is provided at a concentration just below the minimum concentration of enhancer which provides autoagglutination of the particles detectable with the naked eye in the absence of an antigen.

11. A method as claimed in claim 10 wherein the enhancer is PEG, PVP or dextran.

12. A method as claimed in claims 1 or 2 wherein one of the conditions which permits agglutination to occur is that the antibody is present on the surface of the particle at a density sufficient such that the particles are capable of autoagglutinating in a manner detectable with the naked eye in the presence of an enhancer at a preselected concentration.

13. A method as claimed in claims 1 or 2 wherein both the contacting and detecting steps are conducted within a fifteen minute time span.

14. A method as claimed in claim 13 wherein the time span is less than about fifteen minutes.

15. In a method for detecting an analyte in a biological sample by contacting the biological sample with particles coated with an immunological reagent directed towards the analyte, the improvement comprising,

enhancing agglutination of the particles to allow visualization of the agglutination of the particles with the naked eye.

16. A method as claimed in claim 15 wherein the biological sample is sputum.

17. A method as claimed in claim 15 wherein the analyte is a mycobacterium.

18. A method as claimed in claim 15 wherein the enhancer is provided at a concentration just below the minimum concentration which provides autoagglutination of the particles detectable with the naked eye in the absence of an antigen.

19. In an agglutination method involving the contacting of a sample obtained from a biological fluid with particles coated with an antibody specific towards an analyte being tested for and detecting agglutination of the particles as an indication of the presence of the analyte, the improvement comprising, using an antibody directed towards an analyte indicative of mycobacterium in the sample, and contacting and detecting with the naked eye, bacteria present at levels less than 10,000 bacteria per ml of biological fluid.

20. The improvement as claimed in claim 19 wherein the contacting and detecting occur in less than about fifteen minutes.

21. The improvement as claimed in claim 19 wherein the antibody is a monoclonal antibody.

22. The improvement of claim 19 wherein the antibody is a monoclonal antibody directed to a surface antigen of Mycobacterium tuberculosis.

23. In an agglutination method involving the contacting of a biological sample with particles coated with an antibody, the improvement comprising the use of particles coated with a monoclonal antibody at a density sufficient such that the particles are capable of autoagglutinating in a manner detectable with the naked eye in the presence of an enhancer at a preselected concentration.

24. A method as claimed in claim 23 wherein the enhancer is provided at a concentration just below the minimum concentration which provides autoagglutination of the particles detectable with the naked eye in the absence of the appropriate antigen.

25. A particle coated with a monoclonal antibody directed towards mycobacterial antigen, wherein the antibody is present on the particle at a density sufficient such that the particle is capable

of agglutinating in the presence of other such particles in a manner detectable with the naked eye when contacted with a sample obtained from a biological fluid containing less than 10,000 mycobacteria per ml.

26. A particle as claimed in claim 25 wherein the monoclonal antibody is directed towards Mycobacterium tuberculosis.

27. A particle as claimed in claim 25 wherein the monoclonal antibody is directed toward LAM antigen.

28. A particle as claimed in claim 25 wherein the particle is selected from a group consisting of latex beads, red blood cells, glass beads, liposomes, pollen spores, metal oxide particles and carbohydrate particles.

29. A particle as claimed in claim 28 wherein the particle is latex.

30. A particle as claimed in claims 25 or 29 wherein the antibody is on the surface of the particle at a density sufficient such that the particles are capable of autoagglutinating in a

manner detectable with the naked eye in the presence of an enhancer at a preselected concentration.

31. A particle coated with neat ascites containing a monoclonal antibody.

32. A particle as claimed in claim 31 wherein the monoclonal antibody is directed towards Mycobacterium tuberculosis.

33. A particle as claimed in claim 31 wherein the monoclonal antibody is directed towards LAM antigen.

34. A kit useful for clinical, diagnostic testing of biological samples for the presence of mycobactera comprising:

particles coated with a monoclonal antibody directed towards mycobacteria and capable of autoagglutinating in a manner visually detectable with the naked eye in the presence of a preselected concentration of enhancer.

35. A kit as claimed in claim 34 wherein the kit further comprises an enhancer.

36. A kit as claimed in claim 35 wherein the monoclonal antibody is directed towards the LAM antigen.

37. A kit as claimed in claim 34 wherein the monoclonal antibody is on the particles at a density sufficient such that the particles agglutinate in a manner detectable with the naked eye when contacted with a sample obtained from a biological fluid containing less than 10,000 mycobacteria per ml.

38. A kit as claimed in claim 34 wherein the kit further comprises instructions related to the use of the kit in aiding in the diagnosis of an individual suspected of having or being tested for tuberculosis.

39. A kit as claimed in claims 34 or 35 wherein the kit further comprises a slide having defined areas for conducting agglutination reactions.

40. A kit as claimed in claim 35 wherein the enhancer is provided at a concentration just below the minimum concentration which provides autoagglutination of the particles detectable with the naked eye in the absence of an appropriate antigen.

41. A kit as claimed in claims 34 or 35 further comprising a negative control of particles coated with the monoclonal antibody in a manner and

under conditions such that the negative control particles are not capable of agglutinating in the presence of mycobacterial antigen but are capable of agglutinating in the presence of antiimmunoglobulin antibodies.

42. A kit for the clinical, diagnostic testing of biological samples for the presence of mycobacteria comprising:

particles coated with antibody capable of binding to mycobacteria,

reagents for conducting an agglutination assay,

and instructions for using the coated particles and reagents, wherein the assay performed when using the kit permits agglutination visible to the naked eye in about 15 minutes and in more than 50% of the assays when testing biological samples of different individuals having mycobacterial disease.

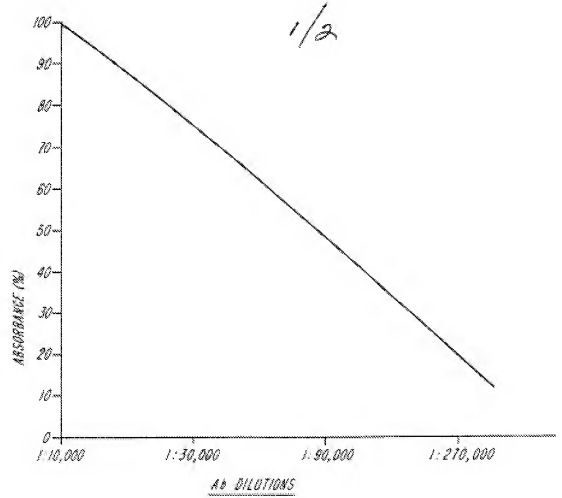

FIG. 1

ENHANCER CONC:	7%	6.5%	6%	5.5%	5%	4.5%	4%	3.5%	3%	... 0.5%	NEG. CNTRL
AMT. OF ENHANCER (µl):	20µl	20µl	20µl	20µl	20µl	20µl	20µl	20µl	20µl	20µl	
BUFFER ASSAY (µl):	40µl	40µl	40µl	40µl	40µl	40µl	40µl	40µl	40µl	40µl	60µl
SESITIZED BEADS (µl):	20µl	20µl	20µl	20µl	20µl	20µl	20µl	20µl	20µl	20µl	20µl
RESULTS:	+	+	+	+	+	+	–	–	–	–	–

+ = AUTOAGGLUTINATION
– = NO AUTOAGGLUTINATION

FIG. 2

2/2

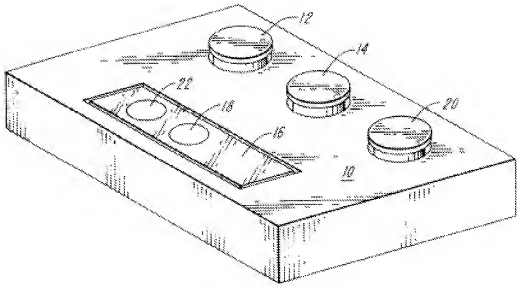

FIG. 3

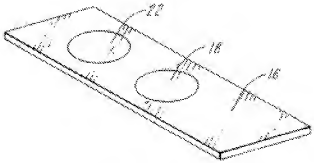

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01131

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[7]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): G01N 33/569, 33/543, 33/546
US CL : 435/7.32; 436/518

II. FIELDS SEARCHED

Minimum Documentation Searched[8]

Classification System	Classification Symbols
U.S.	436/7.32, 962, 967, 975; 436/518, 519, 520, 524, 525, 527, 528, 534, 805, 808, 811, 826

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[9]

DIALOG, APS
Search terms: mycobacteri?(w)tuberculosis(30n)monoclonal; lam or lipoarabinomannan; latex(w)agglutinat?

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X	Journal of Immunological Methods, Volume 129, No. 1, issued 08 May 1990, Cambiaso et al., "Immunological detection of mycobacterial antigens in infected fluids, cells and tissues by latex agglutination", pages 9-14, especially see page 10, right column, "Latex agglutination assay".	15, 17, 42
X	US, A, 4,777,130 (Maes) 11 October 1988, see entire document, especially Example IV, column 7, line 38 to column 8 line 8.	15-17, 42
X/Y	Lancet, Volume 2, issued 01 December 1984, Krambovitis et al., "Rapid diagnosis of Tuberculosis meningitis by latex particle agglutination", pages 1229-1231, especially see "Patients and Methods" on pages 1229-1230.	15, 17, 42/ 1-14, 16, 18-41
X/Y	US, A, 4,812,414 (Warren, III et al.) 14 March 1989, see column 5, lines 7-18 and 49-57, column 11, lines 15-23, column 12, lines 21-33.	15/15
X/Y	US, A, 4,828,981 (Maggio) 09 May 1989, see claim 5.	15/15

* Special categories of cited documents:[15]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
15 APRIL 1992	30 APR 1992
International Searching Authority	Signature of Authorized Officer[20]
ISA/US	Carol E. Bidwell

Form PCT/ISA/210 (second sheet)(May 1986) 8

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X,Y	US, A, 4,851,333 (Goldstein et al.) 25 July 1989, see claims 3 and 14.	15/15
Y	Infection and Immunity, Volume 55, No. 11, issued November 1987, Gaylord et al., "Most Mycobacterium leprae carbohydrate-reactive monoclonal antibodies are directed to lipoarabinomannan", see Abstract and page 2861, left column, lines 5-8.	1-14, 16, 18-41
Y	US, A, 4,362,531 (de Steenwinkel et al.) 07 December 1982, see column 4, lines 20-32.	1-14, 16, 18-41

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter [3] not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.